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(54) Genetic manipulations with recombinant DNA comprising sequences derived from RNA virus.

(57) The invention relates to genetic manipulations of eukaryotic organisms, with recombinant DNA comprising RNA virus derived sequences for protecting such organisms against RNA viruses or enabling inducible or tissue-specific production of foreign proteins/peptides or RNAs. One embodiment of the recombinant DNA according to the invention comprises recombinant DNA, comprising two, 12-1250 base pair long, inverted repeat nucleotide sequences with therebetween at least one nucleotide sequence which is derived from RNA virus which for its replication is dependent upon a viral RNA/RNA polymerase, said RNA virus derived sequence comprising at least cis elements for replication but no gene that codes for viral RNA/RNA polymerase and no gene that codes for viral coat protein. The invention also relates to eukaryotic or prokaryotic cells or organisms which incorporate the recombinant DNA according to the invention. Further the invention relates to a method of protecting such cells or organisms by genetically incorporating recombinant DNA according to the invention.

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nucleic acid in phage envelopes to form virus particles, and cis elements for translation of messenger RNA into protein, in particular coat protein. Examples of trans elements of the genome of RNA viruses are the genes which code for coat protein, transport protein and RNA/RNA polymerase.

An essential element of the invention is that the expression cassette incorporated into the genome of the host leads to transcription of the sequence derived from RNA virus to form a messenger RNA molecule with a panhandle structure. No strict requirements are set to the elements of the expression cassette which regulate this transcription, such as in particular the transcription promoter. The promoter may be, and in many cases will even preferably be, a relatively weak promoter so that the host will be virtually unburdened by this transcription and the transcription products formed in the process. Suitable promoters are known for many organisms. Naturally the expression cassette should also comprise a suitable polyadenylation site, while the expression cassette is flanked at both ends by so-called integration sites enabling integration into the genome of the intended host.

Various experiments have demonstrated that a successful expression in the host of the genetic information incorporated into the genome requires the presence of two, 12-250 base pair long, inverted repeat nucleotide sequences flanking the DNA in-between. These inverted repeat nucleotide sequences may for instance consist of dG-dC base pairs or dC-dG base pairs. The fact that the presence of inverted repeat nucleotide sequences leads to both replication and expression in infected cells of the host, is ascribed to the formation of RNA molecules with a stabilizing panhandle structure (see Van Emmelo et al., Virology 157, 1987, 480-487). For this purpose it is necessary that the inverted repeat nucleotide sequences have a length of at least 12 base pairs. Preferably, however, the inverted repeat nucleotide sequences have a length of at least 15 base pairs. Inverted repeat nucleotide sequences of a length of more than 250 base pairs are not very practical. Preferably, they are not longer than about 50 base pairs.

A further essential feature is the absence of RNA/RNA polymerase (or replicase), or the gene coding therefor, so that the amount of RNA virus-specific messenger RNA present in the cells of the host due to transcription is not further increased. When the RNA virus derived sequence to be incorporated is derived from a satellite virus, such as STNV, this requirement is automatically satisfied because the STNV genome does not contain an RNA/RNA polymerase gene (which explains why the satellite virus depends for its replication on the helper virus, which provides the required RNA/RNA polymerase).

According to the invention it is further of great importance that no viral coat protein is produced and that the nucleotide sequence derived from RNA virus does not contain a gene that codes for viral coat protein.

Finally it is also essential according to the invention that the messenger RNA derived from RNA virus contains at least those elements which in the presence of RNA/RNA polymerase enable replication. In other words, at least cis elements for replication should be present. Depending on the object contemplated, it may be desirable that other elements of the viral genome are present as well. Thus, in particular in the case of protection of the host against virus infections, it is preferable that the genetic information incorporated into the genome of the host also comprises cis elements for transport.

On the other hand, it is not necessary according to the invention that the sequence derived from RNA virus is incorporated into the genome in the sense orientation. The same holds for the orientation of other sequences located between the inverted repeat nucleotide sequences, such as a sequence coding for a ribozyme and a sequence coding for a type-foreign protein/peptide. Owing to the fact that according to the invention both a sense orientation and an anti-sense orientation can be chosen, it is possible to accomplish both a maximum protection under normal, infection-free conditions and an adequate reaction in the case of infection.

Accordingly, the invention primarily provides a recombinant DNA, comprising two, 12-250 base pair long, inverted repeat nucleotide sequences with therebetween at least one nucleotide sequence which is derived from RNA virus which for its replication is dependent upon a viral RNA/RNA polymerase, said RNA virus derived sequence comprising at least cis elements for replication, but no gene that codes for RNA/RNA polymerase, and no gene that codes for viral coat protein.

To be employed, the recombinant DNA according to the invention must be incorporated in an expression cassette for a host to be transformed enabling transcription to take place in the host to form an RNA molecule with a panhandle structure.

Preferably, in a recombinant DNA according to the invention the RNA virus derived sequence contains both cis elements for replication and cis elements for transport.

The invention further includes such a recombinant DNA in which the RNA virus derived s quence also comprises cis elements for packing in coat protein and such a recombinant DNA in which the RNA virus derived sequence also comprises cis elements for translation. In the case of uses where these functions play no role, however, they are preferably absent, because they may hav an adverse effect on the replication efficiency of the RNA formed by transcription. This holds in particular for the use of the invention

formed by transcription may appropriate both in the sense and in the antisense orientation the trans functions for replication and possibly transport of the infecting virus). The invention also offers more possibilities for achieving greater effectiveness, such as the possibility of incorporating one or more ribozyme sequences. The invention combines a smallest possible burdening of the host and a highest possible protection for the host under infection-free conditions with an extremely fast and effective reaction, as soon as an infection by a compatible virus occurs, which reaction gives the host the required protection against the virus, exclusively at locations where that is necessary. The protection the invention offers is of a permanent nature because it is integrated into the genome of the host and, due to its being a minor burden on the host, exerts virtually no selection pressure. A simultaneous protection of the host against several different viruses is a real possibility of the invention owing to the fact that it constitutes virtually no extra burden on the host, i.e. the host need hardly use any energy (the transcription of the DNA inserted requires only very little energy, while in the preferred embodiment no translation into protein is involved). According to the invention the inverted repeat sequences may contain several different RNA virus-specific sequences (i.e. defective replicons of different RNA viruses), and/or several different ribozyme sequences (a ribozyme sequence which is specific for a first virus, a ribozyme sequence which is specific for a second virus, etc.). Naturally, however, these several different RNA virus-specific sequences and several different ribozyme sequences may also be incorporated each within two inverted repeat sequences of their own. In the known methods of virus protection such simultaneous protection against different viruses is not very well possible because the large amounts of entities providing protection, which must be produced continuously throughout the host to ensure effective protection constitute, due to their being a burden, a selective drawback of the host as compared with others of its type and/or are detrimental to its growth and development.

The above-mentioned properties of the recombinant DNA according to the invention are also responsible for its usability as regards methods of producing foreign proteins/peptides and RNAs using genetically modified, prokaryotic and particularly eukaryotic organisms. This concerns recombinant DNA according to the invention in which between the two inverted repeat nucleotide sequences, in addition to the RNA virus derived sequence, a non-viral nucleotide sequence is located which codes for one or more RNAs, and more particularly recombinant DNA according to the invention in which the RNA virus derived sequence comprises at least cis elements for replication and cis elements for translation and between the two inverted repeat nucleotide sequences, in addition to the RNA virus derived sequence, a non-viral nucleotide sequence is located which codes for one or more proteins/peptides.

In virtue of the above-mentioned properties of the recombinant DNA according to the invention the host produces at most a negligible amount of the foreign RNAs or proteins/peptides in the absence of RNA/RNA polymerase, while in the presence of RNA/RNA polymerase a sharp increase of the messenger RNA occurs, which results in a considerable production of the desired RNAs or proteins/peptides. This very strong production can moreover be induced at any desired moment, or be limited to specific tissues of the host by regulating the presence of the RNA/RNA polymerase accordingly.

An inducible production may for instance be accomplished by infecting the genetically modified host (or host cells in a cell culture, for instance) with a compatible virus at the desired moment. Normally, however, this method will not be preferable because it is laborious, the efficiency of the infection may be very variable, the infection mostly takes place at the expense of the host (a possible consequence being a breakdown of the desired product) and/or the use of the virus involves risks. Another, more attractive option, which may also be chosen to accomplish a tissue-specific production consists in a double transformation of the host, wherein the genome of the host is provided not only with the above described recombinant DNA according to the invention but also with recombinant DNA comprising genetic information for a viral RNA/RNA polymerase or an RNA/RNA polymerase construct in an inducible or tissue-specific expression cassette. For this purpose use can be made of known per se inducible (for instance by heat, UV irradiation, or chemicals such as salicylic acid) or tissue-specific (for instance patatin for expression in the tubers of potatoes) promoters.

The phrase "genetic information for an RNA/RNA polymerase construct" refers to gene constructions which may or may not effect coding for another RNA/RNA polymerase. A possible gene construction consists, for instance, of a mutation through which an internal facultative stop codon which may occur in genes coding for RNA/RNA polymerases, is replaced by a sequence not recognizable as a stop codon anymore. Another possibility is a gene construction which consists of a fusion of different RNA/RNA polymerase genes, naturally with the same above-mentioned option of using a mutation of an int rnal stop codon.

The invention is further embodied in eukaryotic or prokaryotic cells or organisms which through genetic engineering are provided with recombinant DNA according to the invention and optionally, through genetic engineering, are also provided with recombinant DNA comprising genetic information for a viral RNA/RNA

antigenic substances, antiviral compounds, anticancer substances, hormones, vitamins, medicines and pharmaceuticals, primary and secondary metabolites.

A further aspect of the invention is recombinant DNA comprising a nucleotide sequence which codes for a viral RNA/RNA polymerase or an RNA/RNA polymerase construct.

More particularly, according to a preferred embodiment, the invention provides such a recombinant DNA comprising the part of the nucleotide sequence shown in Fig. 4 that codes for a viral RNA/RNA polymerase, or constructs derived therefrom, such as a substitution mutant which has sequence TAT instead of the sequence TAG at the positions 656-658 according to the numbering used in Fig. 4, and substitution mutants in which a part of the sequence shown in Fig. 4 is replaced by a corresponding part of another gene which codes for a viral RNA/RNA polymerase.

Such a recombinant DNA in which the nucleotide sequence which codes for a viral RNA/RNA polymerase or an RNA/RNA polymerase construct is located in an inducible or tissue-specific expression cassette constitutes yet another preferred embodiment of the invention.

The invention will now be explained in and by the following Examples.

Example I illustrates how tobacco plants can be protected against infection by TNV through transformation with a replicon derived from STNV. The replicon derived from STNV is a highly defective replicon which does not code for a protein and under infection-free conditions is produced only in a very limited amount, i.e. only a weak transcription of the DNA incorporated into the genome occurs. However, when the plant is infected with TNV, which codes for an RNA/RNA polymerase which is capable of multiplying the STNV derived replicon, massive reproduction of the STNV derived replicon occurs, irrespective of whether the STNV information is incorporated into the genome in sense or anti-sense orientation. As a result, the plant is protected against the infecting virus. This protection is much more effective when the DNA incorporated into the genome also comprises the information for a ribozyme which is directed against mRNA for TNV coat protein.

Example II illustrates how the invention can be used to accomplish an inducible production of a type-foreign protein. As a model for this purpose the beta-glucuronidase gene of E. coli was selected, which was fused with the initiation codon of the STNV coat protein. In the example given the expression was induced by infecting the transformed tobacco plants with TNV.

Example III describes the isolation of the replicase gene of TNV and the construction of a plasmid pSPTNV rep-1, which contains this replicase gene.

Example IV describes expression experiments in which amplification of the messenger RNA by contact with the TNV replicase was effected. The viral replicon contained as foreign DNA a fusion of a part of the gene which codes for the viral coat protein of STNV, and the chloramphenicol acetyl transferase (CAT) gene ofE.coli .

Example I

(a) Recombinant plasmids

Starting from the plasmid pSTNV-413 described by Van Emmelo et al. in Virology 157, 480-487 (1987), a number of new insertion mutants were constructed. The starting plasmid was linearized with Rsa I, which has 9 cutting sites in the STNV genome, by incubating at 28 °C with 0.1 µg enzyme per µg DNA for 15 min. Ligation with the same 14-mer linker as described by Van Emmelo et al: 5′ TCCATGGGAATTCT 3′ - (SEQ ID NO:1) led, among other things, to the insertion mutant pBR STNV N162, which contains the linker with the Nco I site at the 5′ end at position 162 of the STNV genome.

From this insertion mutant and the insertion mutants pBR STNV N198, N322 and E531, already described by Van Emmelo et al., all of which contain the reading frame interfering insertion of the above-mentioned 14-mer linker in the gene coding for coat protein, mutants with a recovered reading frame were constructed. To that end the 14-mer insertion was converted into an 18-mer insertion by cutting with Nco I, filling in the single-stranded ends with Klenow enzyme in the presence of the 4 dNTP's, and religation of the plasmid. Thus the 14-mer insertion is converted into the following 18-mer insertion: 5′ TCCATGCATGG-GAATTCT 3′ (SEQ ID NO:2), in which the Nco I site (CCATGG) is replaced by an Nsi I site (ATGCAT). Thus the insertion mutants pBR STNV N164, N200, N324 and E533 were obtained which theoretically code for a coat protein which is increased by 6 amino acids.

Further the double insertion mutant pBR STNV N164N843 was constructed by isolating from pBR STNV N843 theEco RV fragment of bases 198 to 962 in the STNV genom and substituting it for the

conjugation, the vector is transferred from E.coli SM10 into the Agrobacterium strain GV3100 (pMP90RK), which is used for the plant transformations. The plasmid pMP90RK (Fig. 2; see Koncz and Schell, Mol. Gen. Genet. 204, 1986, 383-396) functions as virulence plasmid. It is derived from the nopaline Ti-plasmid pGV3100 by (1) a combined deletion and insertion mutagenesis, leading to a complete deletion of the T-DNA and to the incorporation of a gentamycin resistance gene (the construction thus obtained is designated as pMP90), and (2) the insertion of a fragment of the P-type plasmid pRK2013 with thereon the genes for kanamycin resistance, the genes acting in trans for transfer and replication of P-type plasmids and the P-type origin of transfer (Figurski and Helinski, PNAS USA 76, 1648 (1979)). The most important properties of this plasmid pMP90RK (see Fig. 2) are:

--- a stable replication only in Agrobacterium
--- compatibility with P-type plasmids
--- it codes for all Ti-virulence functions which are necessary and sufficient for the integration of the T-DNA into the genome of plants during transformation
--- it contains gentamycin and kanamycin resistance genes as genetic markers which are easy to select
--- it complements transfer and replication of P-type mutants by the expression of the pRK2-genes tra1, tra2, and tra3 and trfa
--- it conjugates very efficiently owing to the P-type oriT.

(b) Techniques

The transformation of plants (tobacco SR1) was according to the leaf segment method, described by De Block et al. in EMBO Journal 3, 1681 (1984) and by Horsch et al. in Science 223, 496 (1984). The procedure was as follows:

--- by incisions with a scalpel tobacco leave are wounded and the freshly wounded leave are incubated for 48 h with a 1/50 dilution of a fresh matured culture of the transforming Agrobacteria . This is conducted in liquid MS-plant medium (Murashige and Skoog medium, Gibco)
--- then the leaf fragments are washed twice for 12 h in liquid MS-medium to which claforan (500 µg/ml) is added (claforan is an antibiotic which acts only against bacteria)
--- then the leaf fragments are incubated on solid MS-medium (+ 0.8% agar) to which cytokinin (6-benzyl-aminopurine or BAP, 1 mg/l) and auxin (α-naphthalene acetic acid or NAA, 0.1 mg/1) are added in a ratio which promotes shoot formation at the location of the wounds, kanamycin (100 µg/ml) for the selection of transformed shoots and claforan for the further selection against the transforming Agrobacteria
--- after 4 to 10 weeks transformed shoots grow from this medium which, when they are sufficiently developed, can be transferred to hormone-free medium where they can develop to normal plants with roots.

Callus induction was performed on sterile leaf and stem fragments. For that purpose the fragments were incubated on solid MS-medium (+ 0.8 % agar) to which the cytokinin BAP (0.5 mg/l) and the auxin NAA (1.0 mg/l) were added in a concentration and ratio which promote callus growth.

For octopine tests small particles of plant tissue (about 50 mg) were incubated overnight in a solution of 100 mM L-arginine and 50 mM pyruvate dissolved in liquid MS-medium. After incubation the liquid was removed and the tissue was washed in MS-medium. Then the plant fragment was crushed and centrifuged, and 3 to 5 µl of the extract was separated by paper electrophoresis. This was conducted on Wattman 3MM paper, in a solution of 15 % HAc, 5 % HCOOH and 80 % H_2O. Octopine spots on the paper were made visible under UV lighting after a staining reaction with a fresh mixture of 1 part 0.02 % phenanthrequinone in 95 % EtOH and 1 part 10 % NaOH in 60 % EtOH.

Plasmid infections of cowpea were performed to investigate the infectivity of the STNV mutants. To that end cowpea was inoculated with a mixture of TNV helper virus and chimeric plasmids according to the method described by Van Emmelo.

Northern analyses were performed essentially in the manner described by Van Emmelo. For the analyses of the transformed plants single- and double-stranded RNA were not separated by LiCl precipitation for gel electrophoresis. Besides denaturing glyoxal agarose gels, denaturing formamide agarose gels were used, as described by Lechrach et al in Biochemistry 16, 4743 (1977) and by Maniatis et al in Molecular Cloning: a laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA. In this method the RNA samples ar first denatured at 55° C for 15 min in a mixture of 50 % formamide, 17.5 % formaldehyde, 20 mM morpholino propane sulfonic acid (MOPS) (pH 7.0), 5 mM NaAc and 1 mM EDTA (pH 8.0). Gel electrophoresis was conducted in a 1.2 % agarose gel in 20 mM MOPS (pH 7.0), 5 mM NaAc and 1 mM EDTA (pH 8.0) as a transporting buffer. The furth r treatment of the samples for blotting and hybridization was carried out in the manner described by Van Emmelo.

infections is positive-strand and accordingly corresponds to the genomic RNA (also when no coat protein, no particles and only small amounts of ssRNA are present).

Besides the various pBR STNV plasmids, for the mutants N164.1, N164.2, Brad.1 and Brad.2 also the pPLC and pPCV plasmids were used for plasmid infections, with the same results.

(c3) expression of STNV mutants in transformed plants

Transformations of tobacco SR1 were performed with the following T-plasmids:

pPCV 520
pPCV STNV N164.1 and .2
pPCV STNV N164N843.1 and .2
pPCV STNV Brad.1 and .2

Transformation with pPCV 520 was performed as a check on the transformation experiment itself. Using the infective STNV mutant N164 with a linker insertion in the coat protein gene enables both the coat protein (+6 amino acids) of the mutant and the RNA (by hybridization with the specific linker) of the wild type to be distinguished in the analysis of the results. The mutant N164N843 was selected as replication-deficient genome. In this mutant the insertion N164 is used to identify the coat protein and to distinguish it from the wild type, while the two inserted linkers enable identification of the RNA. The mutant STNV Brad was used for transformation to establish whether the expression of the fusion protein between the STNV coat protein and the bradykinin is amplified by an infection of the transformants with the helper virus TNV. The transformed plants are designated by SR1, followed by the identification of the STNV mutant and further followed by a number used to identify the independent transformants with the same mutant.

The kanamycin resistant plants which after transfer to hormone-free medium with kanamycin developed roots in a normal manner, were increased for further investigation. Of these plants also callus cultures were produced to enable confirmation of the transformation of the kanamycin resistant plants by octopine tests. The point is that the octopine synthase gene in the T-DNA of pPCV 520 is under the control of a tissue-specific promoter of T-DNA gene 5, which is expressed only in stem fragments (very weakly) and in callus tissue, so that the octopine tests are preferably performed on callus tissue. More than 90 % of the kanamycine resistant plants proved indeed to be transformed.

Transformed plants were obtained under sterile growth conditions. For TNV infection small top shoots were first allowed to grow roots in vitro and then transferred to potting soil to be further cultured under greenhouse conditions. The plants were allowed to develop further and infected with TNV when they were sufficiently large. The mechanical infection was performed with a fresh TNV-inoculum, isolated from tobacco SR1. After 72 h the infected leaves were picked, frozen in liquid nitrogen and preserved at -70 °C. Besides the TNV infected leaves, non-infected leaves of the same plant were picked and analysed.

The presence of STNV RNA and coat protein in the various samples were determined by Northern and Western blotting, respectively, Table 2 summarizing the results. The Table reveals that

--- TNV infection of plants transformed with STNV under the control of a constitutive plant promoter and flanked by inverted poly-GC-regions leads to the replication and expression of the STNV genome and to the formation of STNV particles

--- the insertion mutant N164N843 characterized as replication-deficient replicates normally in transformed plants

--- the STNV construction with a fusion between the coat protein and the bradykinin, after infection with TNV, yields replication of the mRNA and amplified synthesis of the fusion protein

--- the detected STNV RNA according to the hybridizations with the various linkers corresponds to the mutant genome which the plants had been transformed with

--- the plants with the STNV genome in the .2 orientation, i.e. they produce the anti-sense mRNA, after infection with TNV also yield STNV replication and expression, without observable differences with the .1 plants

--- the differences in the intensity of the RNA and protein signals do not correlate with the type or the orientation of the mutant STNV genomes, but are typical of the individual character of independent transformants with the same genome.

Owing to the fact that for the assessments only 10 mg plant material was used, neither RNA nor coat protein could be detected in the transformed plants without TNV infection.

Repeat experiments all produced qualitatively and quantitatively similar results. Significant variations were obs rved only for the efficiency of the TNV infection, depending on various factors such as age and the differentiation level of the infected plants, the quality of the inoculum, the temperature, the humitidy, etc.

assumed that the mutants replicate faster and undergo a more efficient infection according as they are smaller (invariably more STNV-RNA is found in the deletion mutant plants than in the wild type plants, and the amount increases according as the mutants become smaller).

The most important observation in the performance of these deletion mutants, however, is their strong interference with the TNV helper virus. When in an RNA infection line STNV deletion mutants are formed, the amount of TNV decreases very sharply. This decrease is so strong after 1 or 2 further infections that without addition of fresh TNV or TNV-RNA to the RNA inoculum no new infections can be carried out any more. Accordingly, the STNV mutants which are formed here by the unnatural RNA inoculations appear to yield a very efficient repression of the TNV infection.

For comparison the in vitro constructed deletion mutant STNV D1 was infected on cowpea by plasmid infection. When this mutant was propagated by RNA inoculation in accordance with the in vivo deletions, its replication proved to be more efficient than that of the original N164 mutant. Like the mutants that form in vivo , STNV D1 represses the TNV replication. This is evidenced by a minor infectivity of an RNA inoculum from these plants.

The deletion mutant STNV D1 constructed in vitro was also transformed to tobacco SR1 to investigate whether the influence on TNV replication would lead to protection of the transformed plants against TNV infection. To determine the effect of the expression of the RNA of the STNV deletion mutant on the development and the course of the infection with TNV, for comparison the following plants were grown under identical conditions as much as possible, infected and further observed:

--- SR1
--- SR1 STNV N164N843.1/1 and /2, with strong and weak expression of STNV, respectively
--- SR1 STNV D1.1/1

In each of these plants 4 leaves were infected which had a development and differentiation of a similar nature. For the infection a fresh inoculum, prepared from TNV-infected cowpea, and a frozen inoculum from infected tobacco were used, both diluted 1/1 (designated inoculum 1 and 3, respectively) and 1/10 (inocula 2 and 4). Two half leaves of the various plants were infected with each of the 4 inocula at similar locations; these leaves were designated a, b, c, and d from the bottom to the top. Thus the following combinations were made: a1 and a2, b3 and b4, c2 and c4, and d1 and d3.

After 72, 96 and 170 h the development of the infection was observed and photographically recorded. Also, after 96 h particles were taken from each leaf and photographed under UV lighting to investigate the hypersensitivity response of the plants. After 96 h the half leaf of each plant with the best developed lesions (b3) was picked. A part of it was frozen and retained for further analysis of RNA and protein composition. The other part was used for the preparation of an inoculum to infect cowpea leaves. For each of the tobacco plants 4 cowpea leaves were infected:

--- with an extract from one single lesion (1 leaf)
--- with an extract from 200 mg leaf tissue (2 leaves)
--- with an 1/5 dilution thereof (1 leaf)

The photographs (not shown) revealed that a great difference occurred in the development of the TNV infection in the various tobacco plants The most important differences were:

--- the number of lesions on the various leaves differed according to the inoculum used (3 > 4 > 1 > 2), with a minor difference between the plants (SR1 > SR1 N164N843.1/1 and /2 > SR1 D1.1/1)
--- the lesions in SR1 D1.1/1 are clearly smaller than in the other plants; tobacco SR1 and N164N843.1/1 differ most, while N164N843.1/2 is in-between. The differences in size of the lesions on one and the same plant depending on the leaf (a > b > c > d) are normal and are associated with the stage of development of the leaves
--- particularly after 170 h it is clear that fairly large portions of the infected leaves of SR1 and SR1 N164N843.1/1 necrotize completely, while the corresponding leaves of D1/1, it is true, show lesions, but otherwise remain normally green
--- UV lighting makes visible fluorescent rings and stains which are a result of the hypersensitivity reaction of the plant. Due to this reaction infected parts of the plant are isolated by a ring of tissue which leads to these parts dying off. For SR1 D1.1/1 these parts are much smaller than for the other plants. This points to a less extensive infection of the leaf.

After 96 h of each plant a part of the half leaf b3 was used to prepare an inoculum for the cowpea leaves to be subsequently infected with. After 72 h very clear differences in the extent of infection of the cowpea leaves were to be observed. The plant which had been infected with the inocula prepared from the deletion plant yielded lesions but each of the leaves was still green. After 72 h the necrotization of the other plants had progressed much further and several of the leaves were so infected that they were alr ady withered completely and almost fell off.

--- th plasmids pPLC STNV GUS.1 and .2 and pPLC STNV GUS dNCO1.1 and .2 were cut with Nsi I (this restriction enzyme cuts right behind the GUS gene and right at the end of the coat protein gene) and ligated. Thus an Nsi I fragment of 468 bp was deleted and the plasmids pPLC STNV GUS dNsi1.1 and .2, and pPLC STGUS.1 and .2 were obtained, respectively. These last two plasmids pPLC STGUS.1 and .2 contain the constructions in which the coat protein gene is entirely substituted by the GUS gene.

Starting from the pPLC plasmids the constructions STNV GUS and STGUS were transcloned as Bam HI-Sal I fragment in the plant vector pPCV 520, behind the pTR1′ promoter. In the process the orientation relative to the promoter is reversed so that, for example, the plasmid pPCV STGUS.2 is formed from pPLC STGUS.1.

Expression in plants was tested by plasmid infections of cowpea with the various STNV-GUS constructions. For that purpose the plasmids pPLC STNV GUS.1 and .2, and pPCV STNV GUS.1 en .2 were used. Cowpea leaves were infected with plasmid and helper virus and picked 3 days after inoculation. A freshly picked leaf was incubated overnight in a 2 mM solution of X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) in 40 mM phosphate buffer, pH 7.4, at 37° C. Then the leaves were fixated in glutaraldehyde (6 h) and decolourized with an ethanol series (from 30 to 95 %). Conversion of the X-Gluc by the glucuronidase leads to the formation of an insoluble blue precipitate.

With the four above-mentioned plasmids four independent infection series were performed on cowpea, in two cases of which a localized blue reaction was observed in the incubated leaves. With pPLC STNV GUS.1 a blue edge was observed at the location of a lesion. Microscopic examination of the fixated material revealed that the stain reaction was localized in the plant cells which were located right at the edge of the lesion. With pPLC STNV GUS.2 the reaction was visible in the form of a 0.3 mm stain. A microscopic examination could not establish whether the reaction was intracellular, because the plant cells had been damaged too severely by infection through bacterial contamination during the incubation with the substrate. However, because the blue colour was preserved during fixation, decolourizing, embedding and cutting, it can be assumed with fairly great certainty that the reaction was plant-specific.

Further, also a transformation of tobacco was performed, using the T-plasmids pPCV STNV GUS.1 and 2. For both constructions transformants were obtained. GUS tests before and after TNV infections of the grown transformants proved to meet the expectations.

Example III

Isolation of the gene for replicase of Tobacco Necrosis Virus

Used as starting material was Tobacco Necrosis Virus (TNV) Kassanis strain A and serotype A, which is capable of multiplying Satellite Tobacco Necrosis Virus (STNV) SV-1 (SV-A serotype). This strain was obtained from Dr. Wieringa Brants, Phytopathologic Laboratory, Baarn, the Netherlands. TNV, free of STNV, was obtained as described by Van Emmelo et al., Virology 157, 480-487 (1987).

TNV virus was increased in Phaseolus leaves (Vigna unguiculata, cowpea), by infection with virus and carborundum. Genomic RNA was prepared from purified TNV particles and converted into cDNA with AMV reverse transcriptase (Boehringer) using random primers. The second strand synthesis was performed by E. coli DNA polymerase I (Klenow fragment) and RNAaseH (Boehringer) according to the method of Gubler and Hoffman (Gene 25, 263-269, 1983). Then T4 DNA polymerase was added to blunt the ends. After cloning into plasmid pSP64 (Promega), cut with Sma I with simultaneous treatment of the ends with phosphatase, the clones were transferred to E. coli MC1061 by transformation and the colonies were tested by colony-hybridization with fragmented ^{32}P ds TNV RNA.

Subgenomic SSRNA prepared from Phaseolus leaves infected with TNV was used for Northern hybridization with the selected TNV clones. The lengths of these subgenomic RNAs were 1.25 kb, 1.5 kb and 3.8 kb, respectively. After agarose gel electrophoresis, the subgenomic RNA was immobilized on a nylon membrane filter (Pall Biodyne) and hybridized with ^{32}P labeled clone DNA. Selected was a clone (pSPTNV127), which hybridized with the two 3′ subgenomic TNV RNAs and contained a fragment located between positions 1900 and 2300 of the TNV RNA. Restriction analysis demonstrated that this fragment contained an internal Hpa II fragment which could be used as a primer for the cDNA synthesis of the replicas gene which is located 5′ terminally on the TNV RNA For the preparatory cDNA cloning, however, use was made of a synthetic oligonucleotide, derived from the sequence of pSPTNV127: 5′ GCTTGTGAG-TATCA 3′ (SEQ ID NO:9). This sequence is complementary to the genomic RNA.

The synthesis of the CDNA was performed as described hereinabove but in the presence of methyl-

CAT-1 and the extract of which was treated with STNV antiserum (the results are not shown here).

3. Multiplication of the STNV-CAT RNA

Two days after inoculation of cowpea leaves with TNV and PSPSTNV CAT-1 DNA, the leaves were picked and the nucleic acid fraction was isolated. DNA was removed with RNAase-free DNAase I and the RNA was analyzed by Northern hybridization with CAT specific, ^{32}P labeled DNA (Tag I fragment from pBR325). Both in the ss RNA and the ds RNA fraction a band of about 1750 nucleotides was detected (the results are not shown here). This clearly demonstrates that the pSPSTNV CAT-1 plasmid DNA is converted by TNV into ss and ds STNV CAT-1 RNA.

Description of the Figures

Fig. 1 shows the map of the plasmid pPCV 520. The T-DNA of this plasmid is indicated by a thick line and is bounded by left hand (LB) and right-hand (RB) border sequences; the unique restriction sites for Sal I and Bam HI can be used for cloning fragments between the plant promoter pTR1' and the polyadenylation signal pAg7, and the restriction sites Bgl II and Bcl I for cloning between pnos and pAg4. The kanamycin resistance gene NPT-II is expressed by PTR2' and pAocs. The octopine gene is expressed with the tissue-specific promoter pg5 and pAocs. The pBR322-sequences with the replication-origin (oriv, colE1) and the ampicillin resistance (Ap) are located in the T-DNA. The pRK2 origin of replication (ori V) and transfer (ori T), the chloramphenicol resistance gene (Cm) and the cohesive ends of lambda (cos) are located outside the T-DNA.

Fig. 2 shows the map of the plasmid pMP90RK. At the location where the Kpn I fragments 9 and 12 of pGV 3100 adjoin one another, the T-DNA inclusive of the border sequences was deleted. The virulence region is entirely intact. By exchange recombination the gene for gentamycin resistance (Gm) and a fragment of pRK 2013, which carries the genes for transfer (tra1, tra2 and tra3), replication (trfa) and kanamycin resistance (Km), were inserted.

Fig. 3 schematically shows the restriction map of the region of the TNV replicase gene and the fragments used for determining the sequence. After cloning into pUC18 the fragments were analyzed in both directions or twice according to the dideoxy method of Sanger to determine the nucleotide sequence.

Fig. 4 shows the complete sequence of the replicase gene of TNV. The amino acid sequence exhibits homology with the replicase genes of CarMV (Carnation Mottle Virus) and TuCV (Turnip Crinkle Virus). The amber stop codon at position 656 is present in the natural genome. For further uses this stop codon was replaced by TAT which codes for the amino acid Tyr. The initiation and stop codons are indicated by

Δ and ∇,

respectively. The amino acid sequence starts at nucleotide position 50 and stops at position 2221.

Fig. 5 schematically shows the construction of the plasmids pSPSTNVCAT1 and pSPSTNVCAT2. Details about this construction are described in Example IV.

Sequence Listing

SEQ ID NO:1
LENGTH: 14 nucleotides
TYPE: nucleotide
TCCATGGGAATTCT

SEQ ID NO:2
LENGTH: 18 nucleotides
TYPE: nucleotide
TCCATGCATGGGAATTCT

SEQ ID NO:3
LENGTH: 36 nucleotides
TYPE: nucleotide
TCCATGCAATCGAGGGTAGGCATGCATGGGAATTCT

SEQ ID NO:4
LENGTH: 36 nucleotides
TYPE: nucleotide
TCCATGCATGCCTACCCTCGATTGCATGGGAATTCT

SEQ ID NO:5
LENGTH: 30 nucleotides
TYPE: nucleotide
GCGGCCGCCCGGGTTTAGTCCTTTTAGGTT

SEQ ID NO:6
LENGTH: 9 amino acids
TYPE: peptide
ArgProProGlyPheSerProPheArg

cell to be transformed enabling transcription to take place in the host cell, forming an RNA molecule with a panhandle structure.

9. Eukaryotic or prokaryotic cells or organisms, which through genetic engineering are provided with recombinant DNA according to claim 8.

10. Eukaryotic or prokaryotic cells or organisms according to claim 9, which through genetic engineering are also provided with recombinant DNA comprising genetic information for a viral RNA/RNA polymerase or an RNA/RNA polymerase construct in an inducible or tissue-specific expression cassette.

11. A method of protecting eukaryotic organisms, such as particularly plants, yeasts and fungi, against an RNA virus which for its replication is dependent upon a viral RNA/RNA polymerase, by performing a genetic manipulation of the organism to be protected, comprising incorporating into the genome of said organism recombinant DNA which in an active expression cassette comprises two, 12-250 base pair long, inverted repeat nucleotide sequences with therebetween an RNA virus derived nucleotide sequence, said RNA virus derived sequence comprising at least cis elements for replication but no gene that codes for viral RNA/RNA polymerase and no gene that codes for viral code protein.

12. A method according to claim 11, in which the RNA virus derived sequence comprises both cis elements for replication and cis elements for transport.

13. A method according to claim 11 or 12, in which the RNA virus derived sequence also comprises cis elements for packing in coat protein.

14. A method according to any one of claims 11-13, in which the RNA virus derived sequence also comprises cis elements for translation.

15. A method according to any one of claims 11-14, in which between the two inverted repeat nucleotide sequences, in addition to the RNA virus derived sequence, at least one nucleotide sequence is located which codes for a ribozyme which is capable of cutting viral RNA or mRNA.

16. A method of producing in an inducible manner one or more proteins/peptides by culturing a prokaryotic or eukaryotic organism, or cells of a prokaryotic or eukaryotic organism, which organism through genetic engineering is provided with recombinant DNA which in an active expression cassette comprises two, 12-250 base pair long, inverted repeat nucleotide sequences with therebetween a nucleotide sequence which is derived from RNA virus which for its replication is dependent upon a viral RNA/RNA polymerase, said RNA virus derived sequence comprising at least cis elements for replication and cis elements for translation, but no gene that codes for viral RNA/RNA polymerase and no gene that codes for viral code protein, and there being located between the two inverted repeat nucleotide sequences, in addition to the RNA virus derived sequence, a non-viral nucleotide sequence which codes for one or more proteins/peptides, in sense or in antisense orientation, and infecting said organism or cells thereof with the virus.

17. A method of producing in an inducible or in a tissue-specific manner one or more proteins/peptides by culturing a eukaryotic organism whose genome, through genetic engineering, incorporates recombinant DNA which in an active expression cassette comprises two, 12-250 base pair long, inverted repeat nucleotide sequences with therebetween a nucleotide sequence which is derived from RNA virus which for its replication is dependent upon a viral RNA/RNA polymerase, said RNA virus derived sequence comprising at least cis elements for replication and cis elements for translation, but no gene that codes for viral RNA/RNA polymerase and no gene that codes for viral coat protein, and there being located between the two inverted repeat nucleotide sequences, in addition to the RNA virus derived sequence, a non-viral nucleotide sequence which codes for one or more proteins/peptides, in sense or in antisense orientation, as well as recombinant DNA which comprises genetic information for a viral RNA/RNA polymerase for an RNA/RNA polymerase construct in an inducible or tissue-specific expression cassette

18. A method of producing in an inducible manner one or more RNAs, such as ribozymes, antisense RNAs and double-stranded RNAs, by culturing a prokaryotic or eukaryotic organism, or cells of a prokaryotic or eukaryotic organism, which organism through genetic engineering is provided with recombinant DNA which in an active expression cassette comprises two, 12-250 base pair long, inverted repeat nucleotide sequences with therebetween a nucleotide sequence which is derived from RNA virus which for its replication is dependent upon a viral RNA/RNA polymerase, said RNA virus derived sequence comprising at least cis elements for replication but no gene that codes for viral RNA/RNA polymerase and no gene that codes for viral coat protein, and there being located between the two inverted repeat nucleotide sequences, in addition to th RNA virus derived sequence, a non-viral nucleotide sequence which codes for one or mor RNAs, in sense or in antisense orientation, and infecting said organism or cells thereof with the virus.

19. A method of producing in an inducible manner or in a tissue-specific manner one or more RNAs, such as ribozymes, antisense RNAs and double-stranded RNAs, by culturing a eukaryotic organism, whose genome through genetic engineering, incorporates recombinant DNA which in an active expression cassette comprises two, 12-250 base pair long, inverted repeat nucleotide sequences with ther b tween a nucleotide

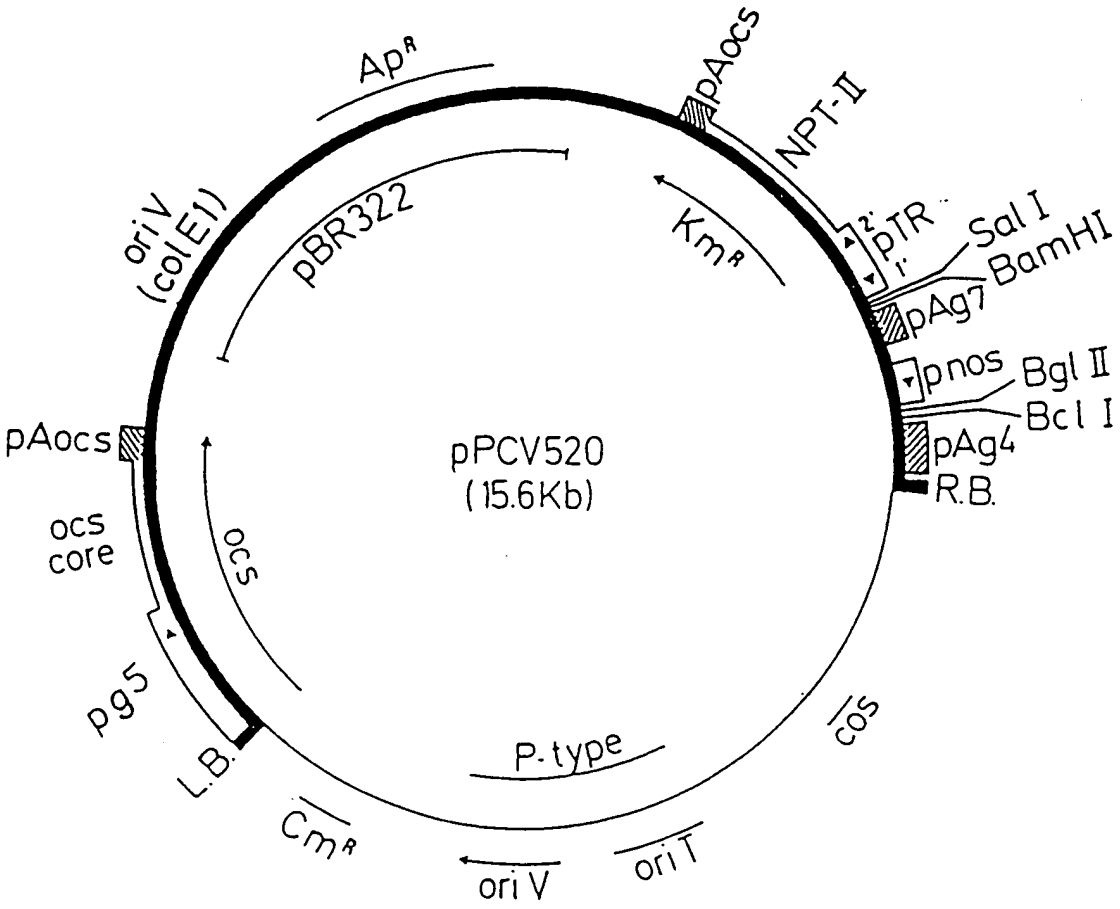
Ap^R
pAocs
NPT-II
ori V
(colE1)
pBR322
Km^R
pTR
Sal I
BamHI
pAg7
pnos
Bgl II
Bcl I
pAg4
R.B.
pAocs
pPCV520
(15.6Kb)
ocs
core
ocs
pg5
L.B.
P-type
cos
Cm^R
ori V
ori T

FIG.1

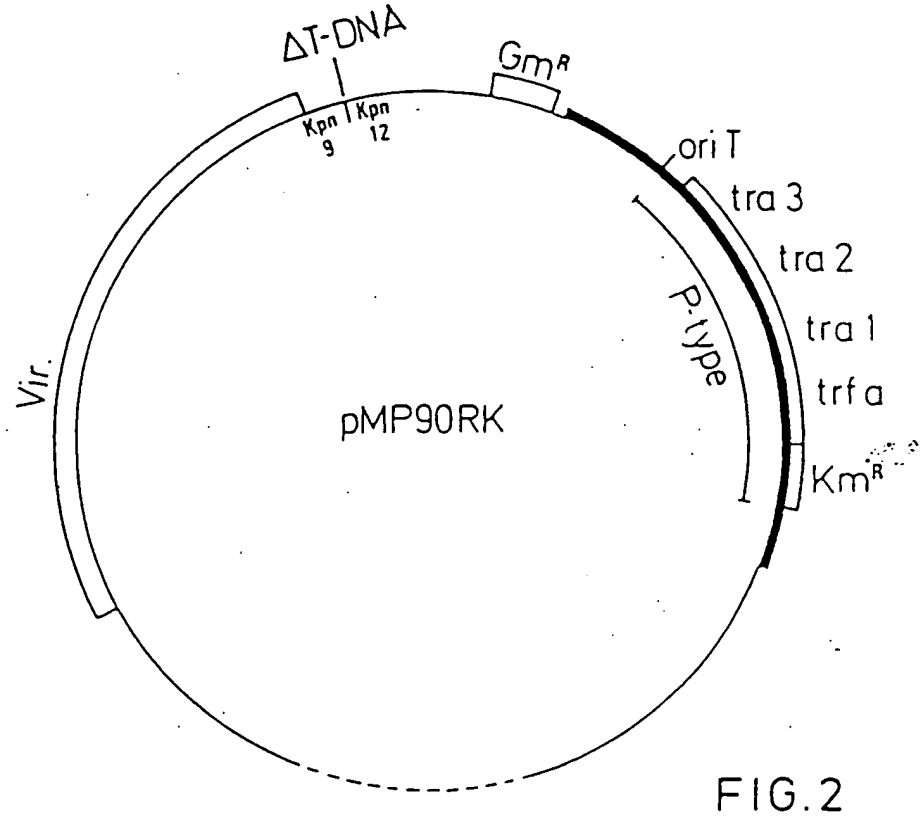
ΔT-DNA
Gm^R
Kpn 9
Kpn 12
ori T
tra 3
tra 2
tra 1
trf a
Km^R
P-type
Vir.
pMP90RK

FIG.2


10 20 30 40 50 60
CCAAGAATACCAAATAGGTGCAAGGCCTTACTCAGCTAAAGAGTCTAAAATGGAGCTACC
GlnGluTyrGlnIleGlyAlaArgProTyrSerAlaLysGluSerLysMETGluLeuPro
70 80 90 100 110 120
AAACCAACACAAGCAATCAGCCGCCGAGGGTTTTGTATCTTTCCTAAACTGGCTATGCAA
AsnGlnHisLysGlnSerAlaAlaGluGlyPheValSerPheLeuAsnTrpLeuCysAsn
130 140 150 160 170 180
CCCATGGAGACGACAGCGAACAGTCAACGCTGCAGTTGTGTTCCAAAAAGATCTTCTAGC
ProTrpArgArgGlnArgThrValAsnAlaAlaValValPheGlnLysAspLeuLeuAla
190 200 210 220 230 240
CATTGAGGATTCCGAGCATTTGGATGATATCAATGAGTGTTTCGAAGAATCTGCTGGGGC
IleGluAspSerGluHisLeuAspAspIleAsnGluCysPheGluGluSerAlaGlyAla
250 260 270 280 290 300
ACAATCCCAGCGAACTAAGGTTGTCGCCGACGGAGCATATGCCCCCGCAAAATCCAATAG
GlnSerGlnArgThrLysValValAlaAspGlyAlaTyrAlaProAlaLysSerAsnArg
310 320 330 340 350 360
GACCCGCCGAGTTCGTAAGCAGAAAAAGCACAAGTTTGTCAAATATCTTGTCAACGAAGC
ThrArgArgValArgLysGlnLysLysHisLysPheValLysTyrLeuValAsnGluAla
370 380 390 400 410 420
TCGTGCCGAGTTTGGATTGCCTAAACCAACTGAGGCAAACAGACTCATGGTCCAACATTT
ArgAlaGluPheGlyLeuProLysProThrGluAlaAsnArgLeuMETValGlnHisPhe

FIG.4

```
      910         920         930         940         950         960
      :           :           :           :           :           :
CAAGGGAGCTTTCCGGACCCTTGATAAGTTTCGTGATCTCTATACTAAAAATAGTTGGCG

 LysGlyAlaPheArgThrLeuAspLysPheArgAspLeuTyrThrLysAsnSerTrpArg

      970         980         990        1000        1010        1020
      :           :           :           :           :           :
TCATACCCCTGTAACTAGTGAACAATTCCTAATGAATTACACGGGCAGGAAACTGACTAT

 HisThrProValThrSerGluGlnPheLeuMETAsnTyrThrGlyArgLysLeuThrIle

     1030        1040        1050        1060        1070        1080
      :           :           :           :           :           :
TTACAGAGAGGCGGTTGATAGTTTGTCGCATCAACCCCTTAGCTCACGAGATGCGAAGCT

 TyrArgGluAlaValAspSerLeuSerHisGlnProLeuSerSerArgAspAlaLysLeu

     1090        1100        1110        1120        1130        1140
      :           :           :           :           :           :
AAAGACATTCGTGAAGGCCGAAAAATTAAACCTTTCTAAGAAGCCTGACCCTGCTCCCAG

 LysThrPheValLysAlaGluLysLeuAsnLeuSerLysLysProAspProAlaProArg

     1150        1160        1170        1180        1190        1200
      :           :           :           :           :           :
GGTCATACAACCTAGATCGCCTCGGTATAACGTTTGTTTGGGCAGGTACCTCCGACATTA

 ValIleGlnProArgSerProArgTyrAsnValCysLeuGlyArgTyrLeuArgHisTyr

     1210        1220        1230        1240        1250        1260
      :           :           :           :           :           :
TGAACATCACGCGTTTAAAACCATTGCCAAGTGCTTTGGGGAAATCACGGTCTTCAAAGG

 GluHisHisAlaPheLysThrIleAlaLysCysPheGlyGluIleThrValPheLysGly

     1270        1280        1290        1300        1310        1320
      :           :           :           :           :           :
GTTTACTCTGGAGCAACAAGGGGAAATCATGCGCTCGAAGTGGAATAAATATGTTAATCC

 PheThrLeuGluGlnGlnGlyGluIleMETArgSerLysTrpAsnLysTyrValAsnPro

     1330        1340        1350        1360        1370        1380
      :           :           :           :           :           :
CGTTGCGGTCGGACTTGACGCCAGTCGTTTCGACCAACACGTGTCTGTTGAAGCACTCGA

 ValAlaValGlyLeuAspAlaSerArgPheAspGlnHisValSerValGluAlaLeuGlu

     1390        1400        1410        1420        1430        1440
      :           :           :           :           :           :
GTATGAGCATGAATTTTATCTCAGAGATTACCCAAATGATAAACAGCTAAAATGGCTGCT

 TyrGluHisGluPheTyrLeuArgAspTyrProAsnAspLysGlnLeuLysTrpLeuLeu
```

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      1930        1940        1950        1960        1970        1980
       :           :           :           :           :           :
GATTCCTGTTATGCAGAATTTCTACCAGATGCTCCAAACTGGCATCCGCCGCACAAAATT
 IleProValMETGlnAsnPheTyrGlnMETLeuGlnThrGlyIleArgArgThrLysPhe

      1990        2000        2010        2020        2030        2040
       :           :           :           :           :           :
CACCAAGACCGGCGAGTTCCAGACGAACGGATTGGGGTATCACTCTAGATTTATGCATAG
 ThrLysThrGlyGluPheGlnThrAsnGlyLeuGlyTyrHisSerArgPheMETHisArg

      2050        2060        2070        2080        2090        2100
       :           :           :           :           :           :
AGTGGCCCGGGTCCCTTCGCCTGAAACCCGTTTATCCTTCTATCTAGCTTTCGGTATCAC
 ValAlaArgValProSerProGluThrArgLeuSerPheTyrLeuAlaPheGlyIleThr

      2110        2120        2130        2140        2150        2160
       :           :           :           :           :           :
ACCAGACCTCCAAGAAGCAATGGAGATCTTCTATGATACTCACAAGCTTGATTTGGATGA
 ProAspLeuGlnGluAlaMETGluIlePheTyrAspThrHisLysLeuAspLeuAspAsp

      2170        2180        2190        2200        2210        2220
       :           :           :           :           :           :
TGTTATCCCGACTGATACCTACCAAGTGTCAGGAGAGCATTTGATCAATGGATTACCAAA
 ValIleProThrAspThrTyrGlnValSerGlyGluHisLeuIleAsnGlyLeuProAsn

      2230
       :
CTGATGTAACGGAGGA
 ---CysAsnGlyGly
    ▼
```

FIG.4

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(54) **Genetic manipulations with recombinant DNA comprising sequences derived from RNA virus.**

(57) The invention relates to genetic manipulations of eukaryotic organisms, with recombinant DNA comprising RNA virus derived sequences for protecting such organisms against RNA viruses or enabling inducible or tissue-specific production of foreign proteins/peptides or RNAs. One embodiment of the recombinant DNA according to the invention comprises recombinant DNA, comprising two, 12-1250 base pair long, inverted repeat nucleotide sequences with therebetween at least one nucleotide sequence which is derived from RNA virus which for its replication is dependent upon a viral RNA/RNA polymerase, said RNA virus derived sequence comprising at least cis elements for replication but no gene that codes for viral RNA/RNA polymerase and no gene that cod s for viral coat prot in. Th inv ntion also relates to eukaryotic or prokaryotic cells or organisms which incorporate the recombinant DNA according to the invention. Further the invention relates to a method of protecting such cells or organisms by genetically incorporating recombinant DNA according to the invention.

EP 0 425 004 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 20 2627

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X,D	VIROLOGY, vol. 157, no. 2, 1987, pages 480-487, Academic Press, Inc.; J. VAN EMMELO et al.: "Expression in plants of the cloned satellite tobacco necrosis virus genome and of derived insertion mutants" * Page 481, left-hand column; figure 1A *	1-4	C 12 N 15/40 C 12 N 5/04 C 12 P 19/34 C 12 N 15/54
X	WO-A-8 908 145 (BIOSOURCE GENETICS CORP.)(08-09-1989) * Example 51 *	20,22	
X	NUCLEIC ACIDS RESEARCH, vol. 13, no. 18, 1985, pages 6663-6677; H. GUILLEY et al.: "Nucleotide sequence and genome organization of carnation mottle virus RNA" * Page 6674, last paragraph; pages 6673, figure 5 *	20	
X	NUCLEIC ACIDS RESEARCH, vol. 16, no. 13, 1988, pages 6097-6111; W.A. MILLER et al.: "Sequence and organization of barley yellow dwarf virus genomic RNA" * Page 6100, paragraph 3; page 6105 *	20	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N
X	CHEMICAL ABSTRACTS, vol. 113, 1990, page 205, abstract no. 127723v, Columbus, Ohio, US; & CN-A-1 033 645 (CHINESE ACADEMY OF SCIENCES et al.) 05-07-1989 * Abstract *	1	
A	EP-A-0 325 066 (INRA)(26-07-1989) * Whole document *	11-14	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	02-05-1991	CUPIDO M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid,

namely claims:

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

x LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions,
namely:

1. Claims 1-19: A method of protecting eukaryotic organisms against an RNA virus, recombinant DNA constructs to be used in this method and cells containing them.

2. Claims 20-22: Recominant DNA constructs encoding viral RNA/RNA polymerases, not to be used in the method of invention 1.

☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid,

namely claims:

☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims,

namely claims:

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 20 2627

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	NATURE, vol. 334, 21st July 1988, pages 196-197; V. WALBOT> et al.: "Plant development and ribozymes for pathogens" * Whole article * ---	5,15	
P,X	VIROLOGY, vol. 177, no. 2, August 1990, pages 699-709; F. MEULEWAETER et al.: "Genome structure of tobacco necrosis virus strain A" * Whole article * -----	20-22	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	02-05-1991	CUPIDO M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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